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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The overall aim of this research is to use a recombinant multiply deleted genomic herpes simplex virus type-1 (HSV-1) based gene transfer vector to test the hypothesis that gene transfer of the glial derived neurotrophic factor (GDNF) or bcl-2 can slow or prevent the apoptotic death of nigral neurons in animal models of PD, and that expression of tyrosine hydroxylase (TH) can improve symptoms.

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4. Introduction.

The overall aim of these studies has been to test herpes simplex virus (HSV)-based gene transer vectors in rodent models of Parkinson disease (PD) in order to develop novel therapeutic agents that might be used to slow the progression or reverse the course of idiopathic PD. The Specific Aims described in the initial proposal were to create genomic HSV-based vectors to express the anti-apoptotic peptide Bcl-2 and the glial cell derived neurotrophic factor (GDNF), to examine the effects of these vectors in preventing characteristic degeneration in the substantia nigra (SN) in the 6-hydroxydopamine (6-OHDA) and MPTP models of PD in rodents, and to compare the time course of the protective effect produced by vectors in which transgene expression was driven by the transiently active human cytomegalovirus immediate early promoter (HCMV IEp) or the HSV latency active promoter (LAP2) element.

5. Body.

In the first three years of these studies we demonstrated that a genomic HSV vector expressing Bcl-2 injected into the SN 1 week prior to 6-OHDA protected the neurons of the SN from degeneration in that model [1], that an HSV-based vector expressing GDNF provided a similar protective effect [2], and that he Bcl-2-expressing and GDNF-expressing HSV based vectors delivered together provide an additive effect in protecting SN neurons from 6-OHDA-induced cell death and loss of tyrosine hydroxylase (TH) immunoreactivity [2]. This additive effectwas different from the response we observed in a different model of neuronal injury (proximal spinal root avulsion) where we found that the Bcl-2 expressing vector protected lesioned motor neurons from degeneration [3] but did not preserve the neurotransmitter phenotype (choline acetyltransferase (ChAT) expression of the lesioned cells [3]. In the spinal root avulsion model the GDNF-expressing vector demonstrated a similar effect, improving cell survival without preserving ChAT expression [4], but the two vectors delivered together acted synergistically to preserve ChAT expression in the surviving lesioned motor neurons. These studies provided proof of principle evidence that HSV-mediated gene transfer an be used to protect neurons from cell death resulting from toxin-induced or traumatic injury, and that in proper combinations are capable of preserving neurotransmitter synthesizing enzyme production and neuronal function.

In last year's progress report we described studies that began to examine the duration of transgene expression from the vector, demonstrating that only a vector in which the LAP2 element was used to drive transgene expression resulted in neuronal protection measured 2 weeks after 6-OHDA injection, when the toxin was applied 30 days after the vector injection. The construction of the vector in which GDNF was under the control of the LAP2 sequence (QL2GD) or under the control of a promoter containing both LAP2 and HCMV elements (QL2HGD) was described in last year's report. In the final year of this project (the subject of the current report) we carried out experiments in three areas: (1) repeated the study to test the protective effect of the GDNF vectors injected thirty days prior to 6OHDA intoxication, following the behavioral recovery and morphologic characteristics out to 60 days after the intoxication (3 months after vector inoculation); (2) characterized a mouse model of chronic systemic MPTP intoxication, which a causes progressive decrement in fine motor coordination, and began to examine the effect of vector inoculation in that model; and, (3) began construction of HSV vectors that will allow inducible expression of transgene expression for application to long term human treatment.

Long-term effect of vector-mediated GDNF expression in the 6-OHDA model

To analyze the efficacy of the new vector we injected adult Sprague-Dawley rats with four different vectors: DHZ.5 (HCMV, lacZ gene; n=5), DHGD (HCMV, GDNF gene; n=3), QL2GD

(LAP2, GDNF gene; n=4) and QL2HGD (HCMV+LAP2, GDNF gene; n=4). On the same day that vectors were injected into the SNc, 3 µl of 2% fluorogold (FG) were injected bilaterally in the striatum to retrogradely label a subpopulation of dopaminergic neurons within the SN that project to the site of the lesion. Thirty days later, 20 µg of 6-OHDA were injected into the striatum ipsilateral to the vector injection. Surgical procedures were performed on a sterotaxic frame (Kopf Instruments) as previously described (Natsume et al., 2001). Amphetamine-induced rotation (5mg/kg) was assessed 14, 30, 45 and 60 days after the nigrostriatal lesions. Two hours after the last amphetamine injection, rats were perfused with 10% buffered formalin phosphate. Forty µm sections were obtained at the level of the SNc to examine FG staining directly. Visualization of dopaminergic cells was done by tyrosine hydroxylase (TH) immunostaining as previously described. The number of surviving FG-positive (FG+) and TH-immunoreactive (TH-IR) cells were counted by an observed blinded to the treatment group. The number of surviving FG+ or TH-IR cells was expressed as a percentage of the similar cells counted on the intact side. An ANOVA test (Bonferroni post-hoc) was used to determine the statistical differences between groups.

Quantification of surviving neurons 60 days after the toxic insult showed that 6-OHDA injection in the striatum caused a profound damage in the SNc ipsilateral to the injection side in the animals that received the control vector (Group I). Only 18.3% of FG+ cells and 34.8% of TH-IR cells survived in this group. Similar percentages of survival were found in the group of animals injected with the short -term action promoter (Group II) where 18.7% of FG+ and 35.1% of TH-IR cells survived in the SNc. In contrast, a high percentage of neurons were spared in the SNc of the group of animals treated with the long-term expression vector (Group III). In this group, 58.7% of FG+ and 75.6% of TH-IR cells were found in the injected side. Interestingly, animals injected with the combined promoter vector (group IV) displayed the same dramatic reduction of FG+ cells (18.5%) as well as TH-IR cells (36.3%) in the SNc as groups I and II. Administration of amphetamine induced ipsilateral rotation to the lesioned side in all animal groups at all times (15, 30, 45 and 60 days post-6-OHDA injection). However, while groups I, II and IV showed an increase in rotations after consecutive amphetamine injections, Group III did not show any worsening in the rotational behavior.

Studies of progressive neurodegeneration in the MPTP mouse model

In order to examine the effect of the vector in a chronically progressive model, we used a a modified version of the staircase test [5, 6] to evaluate skilled forelimb use [7] in mice treated with MPTP. The animals were placed into a plexiglass box containing a double staircase on which food pellets can be presented bilaterally at seven graded levels of reaching difficulty. In order to avoid the possibility of tongue or contralateral paw use, only steps 2 to 5 were baited, with 10 food pellets each on both sides. After two days of food deprivation, the animals were tested for 7 consecutive days until the performance of the intact paw reaches a plateau. After each test the number of pellets taken and eaten was counted, and the average number of pellets eaten in the last three days of testing used as the measure of paw dexterity. The animals were treated with MPTP (4 mg/kg IP, administered 5 days out of 7). Over the course of 20 days these animals showed a progressive decrement in paw reaching behavior (Figure 4). After discontinuation of the MPTP treatment a gradual progressive improvement was noted. Animals sacrificed 1 week after the discontinuation of the treatment nonetheless showed a highly significant correlation between the number of TH-IR neurons in the SN pars compacta and the number of pellets retrieved ($R = 0.820$).

Because it was not possible for us to reliably deliver the vector by stereotactic inoculation directly into the SN in the mice, we injected the vector (5 il) into striatum unilaterally. Injection of either

DHGD or QL2GD directly into striatum 2 weeks after the onset of treatment did not alter the decrement in paw reaching behavior compared to control animals or animals injected with DHZ. However, in animals sacrificed at the conclusion of the experiment, we found that we could not detect vector genomes in the SN in these animals. In a second set of experiments we injected the vector into the striatum in normal animals (N=5) and found that there was efficient transport of the vector to the SN, with vector genomes detectable by real time quantitative PCR using primers specific for HSV ICP27 (Figure 5). We believe that in the intoxicated animals, degeneration of dopaminergic terminals was responsible for the failure of uptake and subsequent retrograde transport of the injected vector. However, it will be possible to inject the vector prior to intoxication, in order to determine whether the vector provides a continuous protective effect to prevent or slow the gradual neurodegeneration that is seen in this model.

Development of an HSV vector with regulatable transgene expression

We have begun construction a set of vectors in which vector–mediated GDNF expression is regulated by incorporation of the Argent™ regulated transcription system (ARIAD Pharmaceutical) into the HSV vector. This system has been constructed based on the human FK506 binding protein (FKBP) and its small molecule ligands, and is described in detail in a publication available through the ARIAD web site (www.ariad.com/regulationkits/images/Reg_Tx-Plasmid.pdf). In brief, the system exploits the ability of specific small molecules to mediate the heterodimerization of FKBPs to the PI3K homologue FRAP. A novel transcriptional activation domain fusion (R_HS) consists of the a 93 amino acid portion of a PI3K homologue termed FRB that is sufficient to mediate binding of the FKBP-rapamycin complex fused to the activator (SH3) consisting of the carboxy terminal 271 amino acids of the p65 subunit of human NF-κB and the activation domain of the human heat shock factor 1. The novel DNA binding construct (ZF3) consists of the DNA binding protein ZFHD1, composed of two zinc finger domains from the human transcription factor Zif268 joined to a homeodomain derived from the human transcription factor Oct-1, fused to 3 FKBP domains. In the presence of the non-immunosuppressive rapamycin analogue AP21967 heterodimerization of R_HS to the ZF3 results in expression of genes placed downstream of the ZFHD1 binding sites. Earlier versions of the same system have been used to regulate transgene expression in vitro [8, 9]and from adenoassociated virus and adenovirus vectors in vivo.

The Argent regulated transcription plasmid kit has been obtained from ARIAD. The coding sequences for R_HS and Z3, separated by an internal ribosome entry sequence (IRES) was excised by digestion of plasmid pC4N2-RhS/ZF3 (ARIAD) with EcoRI and BamHI, and seperately subcloned into shuttle plasmids $p41L_2$ containing the LAP2 promoter, and p41H, containing the HCMV IE promoter, and adequate HSV flanking DNA sequence to enable efficient homologous recombination at the U_L41 gene locus of the HSV vector. These expression/targeting cassettes have been recombined into the U_L41 locus of the HSV vector QOZHG by co-transfection of complementing 7b cells with viral and targeting DNA to replace the lacZ marker gene with LAP2:R_HS IRES Z3 construct to produce the vector QLreg, or with HCMV:R_HS IRES Z3 to produce QHreg. We have confirmed the genetic structure of QLreg and QHreg by Southern analysis and by immunohistochemistry for the transcription factor portion (p65 from NF-κB) of the transgene. These vectors, with the inducible transcription factor located at the U_L41 locus, will be used as starting material for insertion of the GDNF transgene driven by the minimal promoter (ZFHD1 binding sites) at the U_L54 locus.

We have cloned the minimal promoter containing the ZFHD1 binding sites and the IL12 TATA box from $pZ_{12}I$-PL-2 (ARIAD) as an MluI/BamHI fragment into the BamHI site of plasmid pPXE that contains HSV flanking sequences targeting the HSV U_L54 locus, creating plasmid

pPXE-Z12. This construct is a versatile plasmid with unique HinDIII and BglII sites situated between the minimal promoter and the polyadenalyation elements for cloning desired transgenes into that can then be efficiently recombined into the U_L54 locus of QLreg or QHreg as desired. The coding sequence for GDNF are currently being cloned into the HinDIII site of pPXE-Z12 and will be recombined into QHreg and QLreg. The new recombinants (QLregGD and QHregGD) will be isolated by three rounds of limiting dilution purification and the genetic structure confirmed by Southern blot. Functional analysis will initailly be performed in vitro by assesing rapalog induction of GDNF production following infection of Vero cells and primary DRG neurons.

6. Key Research Accomplishments

a. Demonstrated that using the LAP2 promoter element to drive expression of GDNF from an HSV vector provides a protective effect against 6-OHDA-induced dopaminergic cell degeneration measured 3 months after vector inoculation.

b. Developed a mouse model of progressive dopaminergic cell degeneration in the SN, in which measures of manual dexterity (paw-reaching) correlate with cell loss in the SN.

c. Progress in the development of an HSV vector with regulatable transgene expression.

7. Reportable Outcomes

Publications in peer-reviewed journals:

Yamada, M., Natsume, A. Oligino, T., Mata, M., Goss, J.R., Glorioso, J.C., and **Fink, D.J.** Herpes simplex virus vector-mediated expression of Bcl-2 protects spinal motor neurons from degeneration following root avulsion. Experimental Neurology 168:225-230, 2001.

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Iwai, M., Harada, Y., Ishii, M., Kashima, K., Mazda, O., Tamura, M., Wolfe, D., Goins, W.F., and **Glorioso, J.C.** Suicide gene therapy of human hepatoma and its peritonitis carcinomatosis by a vector of replicative-deficient herpes simplex virus. Biochem and Biophys Res Com 291:855-860, 2002.

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Presentations:

David Fink, M.D.

Glaucoma Foundation Eighth International Think Tank, "Preventing Cell Death with HSV-based vectors"
New York, NY, July, 2001

Gene Therapy and Molecular Biology International Conference, Plenary Talk, "Modifying Neural Structure and Function with HSV-based vectors"
Corfu, Greece, August, 2001

Pain and Neurosensory Mechanisms Branch, NIDCR, NIH. "Gene transfer for the treatment of pain: Studies with HSV-based vectors"
Bethesda, MD, October, 2001

Canji Inc. (division of Schering), "Experience with HSV-mediated gene transfer to the peripheral nervous system"
San Diego, CA, November, 2001

Society for Neuroscience, Annual Meeting, Moderator, "Neuromuscular Disease II" Scientific Session
San Diego, CA, November, 2001

New York Academy of Science, Viruses as Gene Delivery Vectors Meeting, "Herpes Simplex virus-based vectors for central and peripheral-based nervous system applications"
New York, NY, December, 2001

Neuroscience Graduate Program, University of Cincinatti, "A good herpes infection: Using HSV to treat diseases of the nervous system"
Cincinnati, OH, January, 2002

American Academy of Neurology, Breakfast Seminar, Organizer and Speaker "Current Status of Gene Therapy"
Denver, CO, April 2002

Gene Therapy and Vaccines, Bio-Europe 2002, "Herpes-mediated gene transfer for pain and neuropathy"
Rome, Italy, May, 2002

American Society for Gene Therapy, Workshop Co-Organizer and speaker, "Herpes mediated gene transfer to the peripheral nervous system"
Boston, MA, June, 2002

American Society for Gene Therapy, "HSV vectors: Promoter Choice for CNS Applications" in Workshop: "Altering Vectors to Improve CNS Expression and Delivery".
Boston, MA, June, 2002

Joseph Glorioso, Ph.D.

Invited Speaker - AACR/NCI/EORTC International Conference, Miami Beach, FL - "Herpes virus delivery of anticancer genes" - October 2001

Invited Speaker - European Society of Gene Therapy Annual Meeting, Antalya, Turkey - "Applications of HSV gene vectors to nervous system disease" - November 2001

Keynote Speaker - Cancer Workshop on The Genomic/Proteomic Revolution & Cancer, Naples, FL - "Herpesvirus Vectors & Gene Therapy of Cancer" - February 2002

Invited Speaker - Research Seminar, Dept. of Ophthalmology, University of Pittsburgh School of Medicine, Pittsburgh, PA - "HSV vectors gene therapy of peripheral nervous system disease" - February 2002

Invited Speaker - Cellular & Molecular Treatments of Neurological Diseases Conference, Ameican Academy of Arts & Sciences, Cambridge, MA - "Engineering virus vectors for PNS applications" - March 2002

Invited Speaker - Genetic Enhancement of Athletic Performance meeting, Cold Spring Harbor Laboratory, NY - "Detection of gene transfer and genetic approaches to pain control" - March 2002

Invited Speaker - Frontiers in Gene Therapy meeting, Stanford University, CA - "HSV vectors for treatment of peripheral nervous system" - April 2002

Invited Speaker - CNS Seminar Series, Roche Bioscience, Palo Alto, CA - "HSV vectors for treatment of peripheral nervous system" - April 2002

Invited Speaker - Cardiovascular Cell and Gene Therapy Conference, Boston/Cambridge - "Treatment of peripheral nervous system disease using herpesvirus gene vectors" - April 2002

Co-Chair and Speaker - American Society of Gene Therapy 5th Annual Meeting, Boston MA - Workshop entitled: Herpesvirus vectors for gene transfer to the peripheral nervous system in vivo. Presentation entitled: "Treatment of peripheral neuropathy using HSV gene vectors." - June 2002

8. Conclusions

This is the final report for this 4 year grant. We have largely accomplished the aims initially proposed. The experiments performed to date demonstrate that HSV-mediated gene transfer may be used to protect DA neurons of the SN against degeneration, and that with the proper choice of promoter a prolonged effect may be achieved. We have developed a mouse model that will be useful to define the parameters of the protective effect, and are constructing a vector with regulatable gene expression that would be appropriate ultimately for use in clinical trials. We are in the process of preparing a submission to the NIH and to the Michael J Fox Foundation to continue this work towards the development of an effective gene transfer therapy for PD.

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FIGURE 1

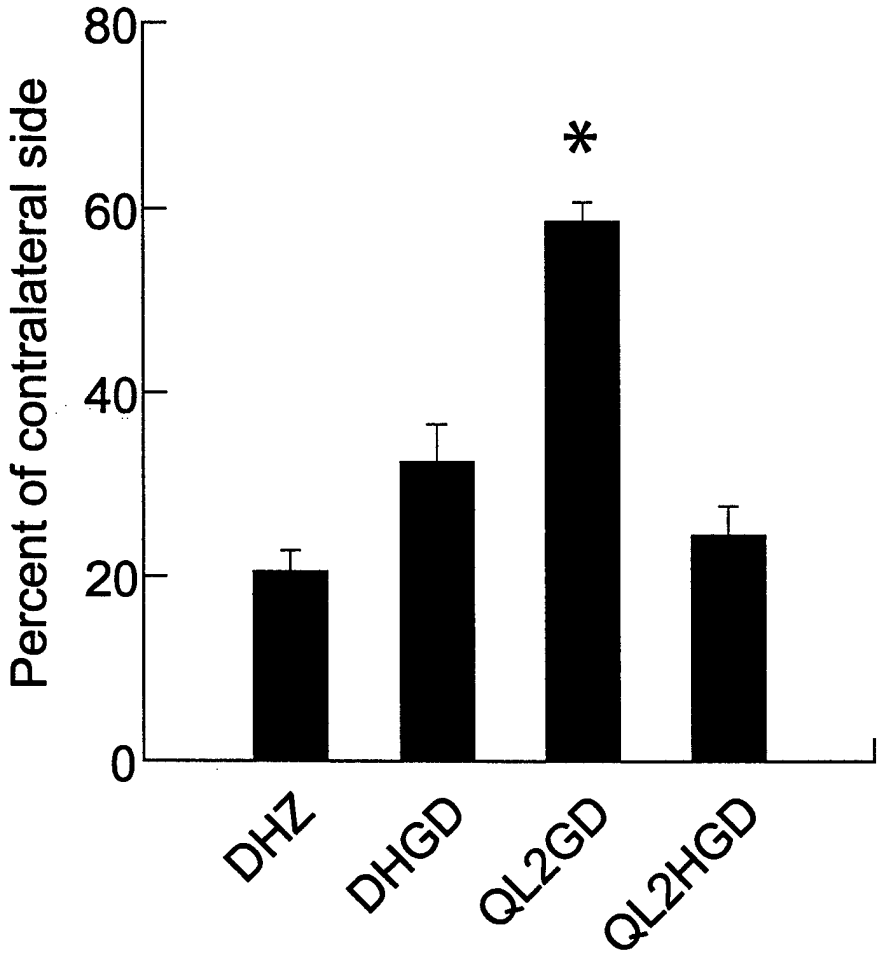

Percent of contralateral side
80
60
40
20
0
*
DHZ
DHGD
QL2GD
QL2HGD

FIGURE 2

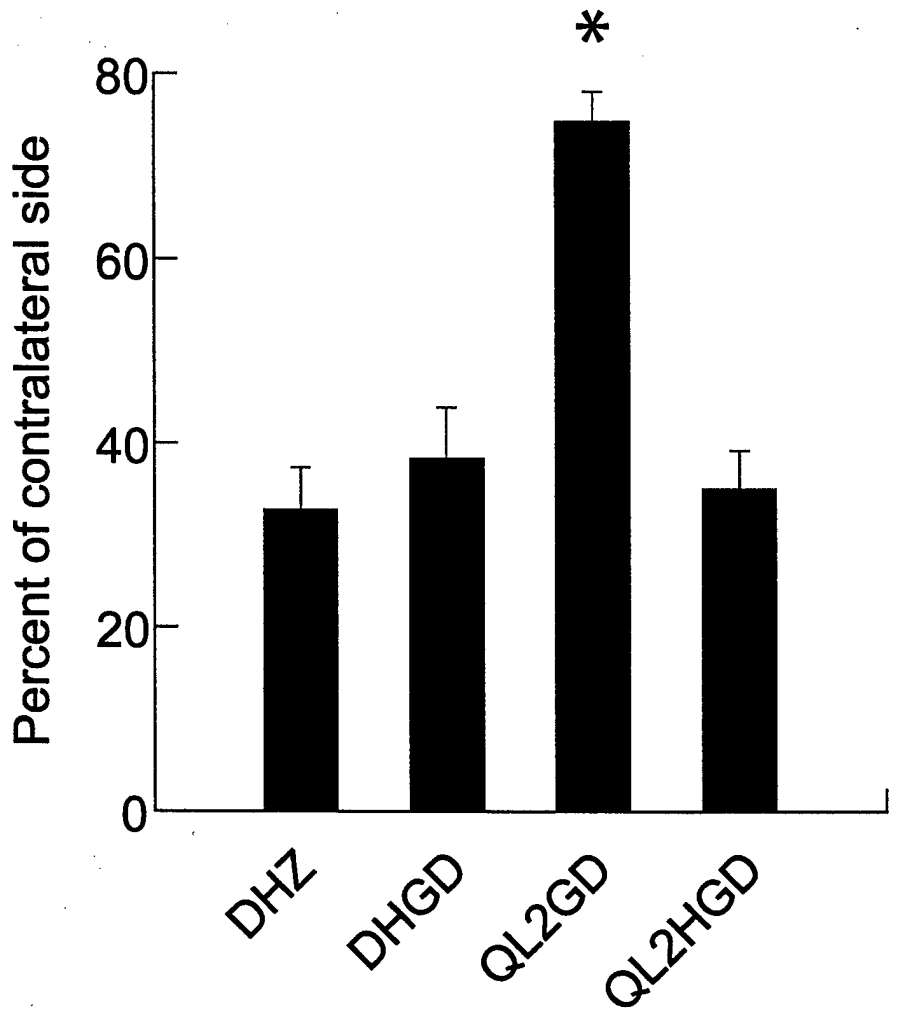

*
80
60
40
20
0
Percent of contralateral side
DHZ
DHGD
QL2GD
QL2HGD

FIGURE 3

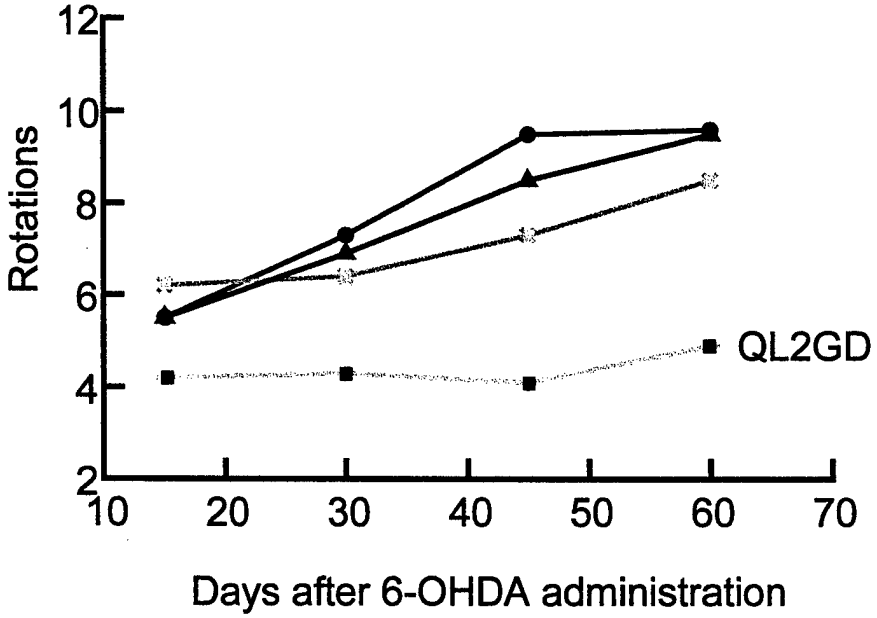
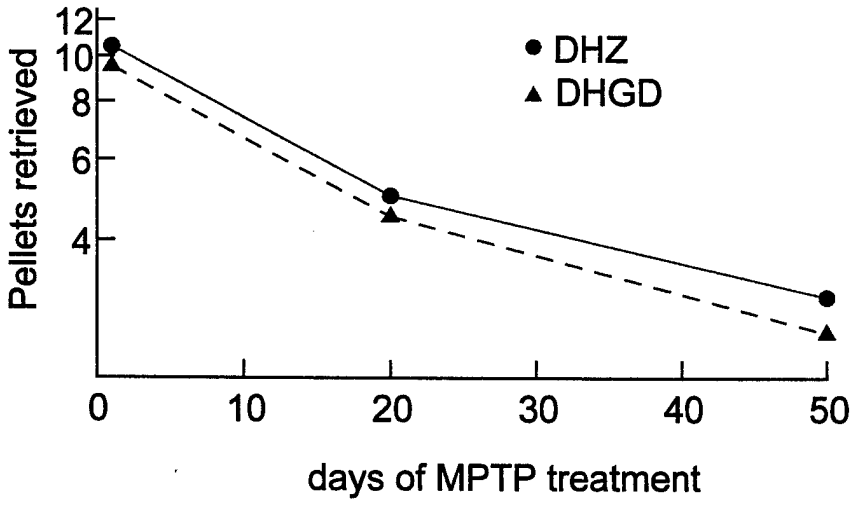

12
10
8
6
4
2
Rotations
QL2GD
10
20
30
40
50
60
70
Days after 6-OHDA administration

FIGURE 4


Pellets retrieved
12
10
8
6
4
0
10
20
30
40
50
days of MPTP treatment
DHZ
DHGD

Personnel Listing
DAMD17-98-1-8626
Dr. Joseph C. Glorioso, Principal Investigator

NAME	ROLE
Joseph C. Glorioso	PI
David Fink	Co-PI
William Goins	Co-PI
Marina Mata	Co-PI
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Changsook Hong	Res. Associate
Waris Shah	Res. Associate
Xiao Peng Zhang	Res. Associate
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Jeremy Somers	Technician
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